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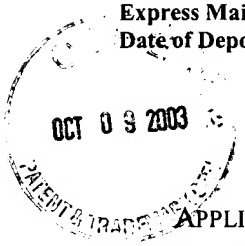
Express Mail Label No. EL 991 159 902 US
Date of Deposit: October 9, 2003

Attorney Docket No.: 21465-501 CIP2



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS :	Rothberg et al.		
SERIAL NUMBER :	09/814,338	EXAMINER :	Young J. Kim
FILING DATE :	March 21, 2001	ART UNIT :	1637
FOR :	METHOD OF SEQUENCING A NUCLEIC ACID		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Transmitted herewith for filing in the present application are the following documents:

1. Declaration of Marcel Margulies under 37 C.F.R. § 1.132 (4 pages);
2. Exhibits 1-3 for Declaration (21 total pages);
3. Return Postcard.

If the enclosed papers are considered incomplete, the Mail Room and/or the Application Branch is respectfully requested to contact the undersigned at (212) 935-3000, New York, New York.

The Commissioner is authorized to charge any fees that may be due, or to credit any overpayment, to the undersigned's account, Deposit Account No. 50-0311 Ref. No. 21465-501 CIP 2. Please address all correspondence to customer number **35437**. A duplicate copy of this transmittal letter is enclosed herewith.

Respectfully submitted,

Ivor R. Elrifi, Reg. No. 39,529
Caryn DeHoratius, Reg. No. 45,881
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Dated: October 9, 2003

Express Mail Label No.: EL 991 159 902 US
Date of Deposit: October 9, 2003

Attorney Docket No. 21465-501 CIP2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Jonathan M. Rothberg, et al.		
ASSIGNEE:	CuraGen Corporation		
SERIAL NUMBER:	09/814,338	EXAMINER:	Young J. Kim
FILING DATE:	March 21, 2001	ART UNIT:	1637
FOR:	METHOD OF SEQUENCING A NUCLEIC ACID		

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MARCEL MARGULIES UNDER 37 C.F.R. §1.132

I, MARCEL MARGULIES, declare and state that:

1. I am Vice President of Engineering, at 454® Life Sciences, the exclusive licensee of this application. My previous employment includes Director of New Technology Research at Perkin-Elmer's Instrument Division in Norwalk, CT, and Associate Director of the Hubble Space Telescope project.

2. I earned my B.Sc. in Engineering from the Free University of Brussels, in Belgium, and a Ph.D. in theoretical physics from Columbia University.

3. I have reviewed the instant application and the August 18, 2003 Office Action in this case.

4. It is my opinion that the claimed invention represents the first massively parallel, solid-phase, whole-genome sequencing platform, which is vastly superior to previous sequencing technology for at least the reasons set out below.

5. Although DNA sequencing was performed by Gilbert and Sanger as early as 1977, the apparati claimed in the instant application are the first to allow rapid massively parallel sequencing (e.g., of whole viral or bacterial genomes). Traditional methods for genome sequencing have been slow, expensive, laborious, and industrial-scale, since they involve individually preparing and sequencing samples (DNA fragments) of the genome. The Human Genome Project, for example, required approximately 12 years, $2.7 billion dollars, and 60 million samples to complete.

6. In contrast, the substrates and apparati claimed in the instant application provide a massively parallel, scalable platform that dramatically reduces the time, cost, sample preparation, and space required for genome sequencing. Instead of individually preparing and sequencing each sample, the claimed substrates and apparati allow parallel sequencing of thousands (or hundreds of thousands) of samples.

7. Recently, the claimed substrates and apparati were used to sequence the entire adenovirus genome (approximately 30,000 base pairs) contained on an expression vector in less than one day (see NY Times article, Ex. 1). The entire sequencing process from sample preparation to data analysis was accomplished in less than one day, and provided over 99% genome coverage. The resulting adenovirus sequence was published in GenBank under Accession Nos. AY370909, AY370910, and AY370911 (Ex. 2).

8. To generate this sequence information we fabricated preferred commercial embodiments of the claimed substrates and apparati. In these preferred embodiment, the claimed substrate is termed a "PicoTiter Plate". The PicoTiter Plates used to generate the data referred to in Exs. 1, 2 and 3 were cavitated fiber optic wafers formed from a fused bundle of a plurality of individual optical fibers (as recited in the pending claims). Specifically, we fabricated PicoTiter Plates by

acid etching the top surface of fiber optic wafers to form wells with diameters between 39 and 44 μm (as recited in the pending claims). The fiber optic wafer has a thickness of about 2.0 mm (also as recited in the pending claims). In addition, we fabricated the wells on PicoTiter Plates with depths ranging from 26 to 76 μm (i.e., from between one half the diameter of an individual optical fiber and three times the diameter of an individual optical fiber, as recited in the pending claims). Finally, we loaded the wells with nucleic acid template and a beads with pyrophosphate sequencing reagents attached thereto (as recited in the pending claims). Sequencing by synthesis was then performed as described in the specification, and using the claimed apparatus to flow sequencing reagents over the PicoTiter plate.

9. In further experiments, the apparatus of the instant application was used to sequence a segment human chromosome 12 (approximately 170,000 base pairs) contained on an artificial chromosome vector (Ex. 3). With the apparati, a one-day sequencing run produced sufficient shotgun sequence coverage of the chromosome 12 clone (Ex. 3, p. 6). A single sequencing run obtained 85% genome coverage and 98% consensus accuracy (Ex. 3, p. 3). These results were presented at the15th Annual Genome Sequencing and Analysis Conference, held on September 21-24, 2003 (Ex. 3, p. 1).

10. The substrates and apparati claimed in the instant application therefore fulfill a long-felt but unmet need for rapid, whole-genome analysis of viral and bacterial pathogens (e.g., ¶ 7 above). Such analysis is critical for biodefense, drug discovery, and the identification of emerging pathogens. More than this, the claimed apparati solve the long-standing problems with analysis of large genomes, such as in humans (e.g., ¶ 9 above). Solutions for large-genome sequencing are vital for drug development, early diagnosis, and faster clinical interventions.

11. For these reasons, in my opinion, the claimed substrates and apparati represent a significant advancement in the field as the first massively parallel, solid-phase, whole-genome sequencing platform that can be scaled for viral, bacterial, and even human genomes.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

Dated: 10/8/03

Signed: _____
Marcel Margulies

Exhibit

Company Says It Mapped Genes of Virus in One Day

By ANDREW POLLACK

A small company developing a novel method of sequencing genes said yesterday that it had determined the entire genetic code of a virus in a single day.

The company, 454 Life Sciences, said it was the first time that the entire genome of an organism had been sequenced using an unconventional technique and demonstrated the feasibility of its technology, which it said might eventually allow gene sequencing to be done faster and less expensively.

"It's a real threshold moment," said Richard Gibbs, director of the genome-sequencing center at Baylor College of Medicine in Houston and an adviser to the company. "This is going to be big."

Other scientists were more skeptical, saying that sequencing a virus, which has a tiny genome, is trivial and that there are aspects of the

technology that might make it difficult to do more complex organisms.

"I think doing a whole bacterium will be a challenge," said Edward M. Rubin, director of the Joint Genome Institute, a Department of Energy sequencing center in Walnut Creek, Calif. Bacteria are the next level up in complexity after viruses.

454, based in Branford, Conn., is one of several companies racing to improve gene sequencing. Some scientists say it might one day be possible to sequence an entire human genome in a few days for as little as $1,000 so that each person could have his or her genome for use in predicting susceptibility to disease and choosing the most appropriate medicines. The Human Genome Project, which first sequenced a human genome, took years and cost tens of millions of dollars.

Still, the thousand-dollar genome is years away. The human genome, which consists of more than three billion letters of the genetic code, is about 100,000 times the size of the adenovirus that 454 sequenced, which has a genome of about 33,000 letters.

454 is majority owned by CuraGen, a genomics company. The name 454 was the code name by which the project was referred to at CuraGen and the numbers have no special meaning, Richard F. Begley, chief executive of 454, said. Some of the company's technology is licensed from Pyrosequencing A.B., a Swedish company. Mr. Begley said the company hoped to start offering a sequencing service by the end of the year and to begin selling machines about six months after that.

Sequencing is usually done in big sequencing centers with dozens of machines and dozens of people. Dr. Begley said 454's technology would pave the way for the "personalized genome center" in which a single scientist could do sequencing on one

machine in a small room.

The company's technique does more sequencing in parallel than the conventional technique, he said. Another significant time-saving development, he said, comes in preparing the samples for sequencing. The 454 technique, he said, requires one sample preparation for each organism compared with dozens and even thousands for the conventional technique.

Scientists differed on whether sequencing a virus in a day was truly fast. The Department of Energy once did 30 bacteria in 30 days. The virus that causes SARS was sequenced in six days, but it might be misleading to compare that effort with 454's.

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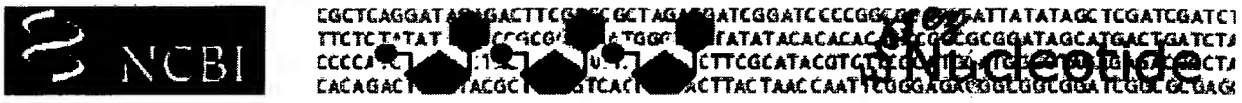

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REFERENCE   2  (bases 1 to 30091)
  AUTHORS   Lohman,K.
  TITLE     Direct Submission
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Human BAC Sequence and Assembly by 454 Life Sciences' Sequencing by Synthesis Method

Lei Du[1], David A. Wheeler[2], George Weinstock[2], Richard Gibbs[2], Gina Costa[1], John Leamon[1], Jan Berka[1], Srinivasan Maithreyan[1], Gary Sarkis[1], Kenton L. Lohman[1]

[1] 454 Life Sciences, Branford, CT

[2] Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX

Abstract

454 Life Sciences has developed proprietary methods for massively parallel DNA sequencing. We have applied this technology to re-sequencing and mapping human BAC clones to their precise chromosomal locations. This preliminary data shows the efficacy of the technology to rapidly sample and characterize subsets of sequence spanning an entire genome or a specific chromosomal location. The novel DNA sequencing method consists of three steps: template preparation, solid phase amplification, and solid phase DNA sequencing. Several thousand to several hundreds of thousands of DNA sequencing reactions are performed simultaneously on glass plates containing 300 thousand to 1 million 75 picoliter volume wells. Average read length of each fragment is consistently greater than 50 bases. The starting point for genome sequencing involves a single template preparation and an absence of a bacterial plasmid cloning step, thus greatly reducing costs and increasing the throughput of our system. In addition, we are completing development of a new software algorithm for *de novo* whole genome assembly. Sequencing results from human BAC clones will be presented and discussed.

Experimental Procedures

Our novel methodology requires only a single sample preparation per genome, utilizes simultaneous clonal amplification of shotgun fragments in sub-nanoliter microreactors, without the use of time-consuming cloning steps. The product of each microreactor is driven to anticipate by a concomitant solid support. The captured DNAs are delivered to wells on the PicoTiterPlate™ and sequenced on 454 Life Sciences' sequencing platform. The details of these steps are illustrated in Figure 1.

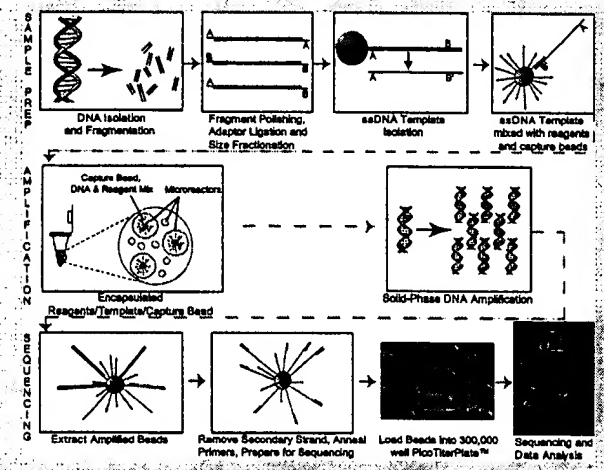


Figure 1. Streamlined template preparation and amplification process.

1) BAC DNA from clone RP11-418C2 was fragmented to sub-kilobase lengths.
2) The fragment ends were polished and adaptor oligo A was ligated onto each fragment, and the sample was size fractionated, resulting in products under 500 bases in length.
3) One strand of these double-stranded products was bound to microparticles, and the free strand was released as template for the subsequent amplification reaction.
4) Amplification was conducted in a single emulsion preparation, encapsulating the reaction reagent mix, a single DNA capture bead and template on a 40 to 100 picoliter microreactor.
5) The unique template molecules retained in each individual microreactor was amplified and immobilized on the respective DNA capture bead.
6) The DNA capture beads were extracted and the template DNA was prepared for use on the 454 sequencer.

Data Analysis

The 454 sequencer generates raw traces for each microreactor, and produces sequence reads in FASTA format using a proprietary basecaller program. Adaptors and low quality reads are removed and repeats masked before mapping and assembly.

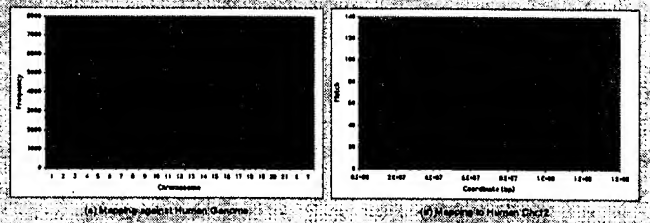
Human Genome Mapping:
Each masked read was mapped against the human genome (NCBI build 33) using BLAT and the mapped reads (>95% identity) are recorded for each chromosome.

BAC Assembly:
Each sequence was mapped against the reference BAC sequence (RP11-418C2) using a proprietary alignment algorithm and the resulting alignment was recorded. For sequences that map to the genome with >90% accuracy, the software generates a list of individual bases found at a given position in the reference genome. The consensus base for each location was computed by averaging all mapped bases. This consensus sequence was then compared with the reference sequence to calculate total accuracy and coverage.

We also mixed 3x oversample of reads (950 sequences) generated from conventional Sanger method with reads generated from the 454 sequencer and assembled with Phrap using default parameters.

Results and Discussion

Human Genome Mapping:
Out of 8561 mapped reads, 7153 are mapping to human chromosome 12 (Fig. 2a). Of these, 7058 reads map to the expected location within chromosome 12 (Fig. 2b). The coordinate boundaries for clone RP11-418C2 in NCBI build 33 are 11,818,492-11,986,440, whereas boundaries on the 7058 read stack are 11,816,616-11,986,511. We also mapped these reads to the mouse genome, and located the BAC to the syntenic region on mouse chromosome 6 (data not shown).



(a) Mapping against Human Genome (b) Mapping to Human Chr12

Figure 2. Sequence mapping against human genome and within chromosome 12.

BAC Assembly:
In a separate sequencing run, we generated 97,193 raw reads from this BAC clone. After, adaptor removal, repeat masking and quality trimming, 39,900 reads were assembled against the reference sequence (Fig. 3). Genome coverage is 99% and consensus accuracy is 98%. Average read length is 84 bases.

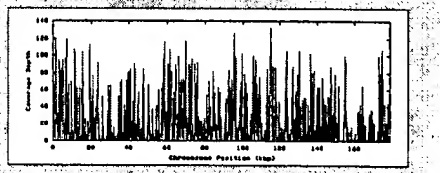


Figure 3. Frequency of assembled reads across BAC sequence length.

Phrap Assembly:
Sanger reads alone generated 25 major contigs (>2 kb) with a 76% mapping efficiency, whereas Sanger and 454 reads combined produced 18 major contigs with a 83% mapping efficiency. 454 reads were able to join and extend Sanger contigs into much larger stretches (Fig. 4).

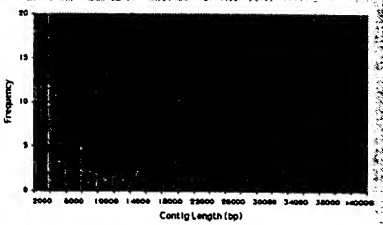


Figure 4. Distribution of Phrap contigs from Sanger only and Sanger+454 reads.

Conclusion

We have demonstrated in this study that 454 Life Sciences' novel sequencing methodology is capable of producing sufficient shotgun sequence coverage of a BAC clone in a single run (done within 1 day). The reads can be used to map its precise location in the genome, as well as assembling into contigs based on reference sequence. This is a useful tool for whole genome mapping and sequencing.

We also showed that by combining conventional Sanger method with 454 technology, we achieve a better *de novo* assembly outcome for whole genome shotgun sequencing.

We are continuing to develop our quality scoring and trimming algorithm. We have completed phase one of our proprietary fragment assembler designed to take advantage of the raw trace signals produced by our sequencing-by-synthesis method. This assembler will be available as part of 454's commercial sequencing instrument.

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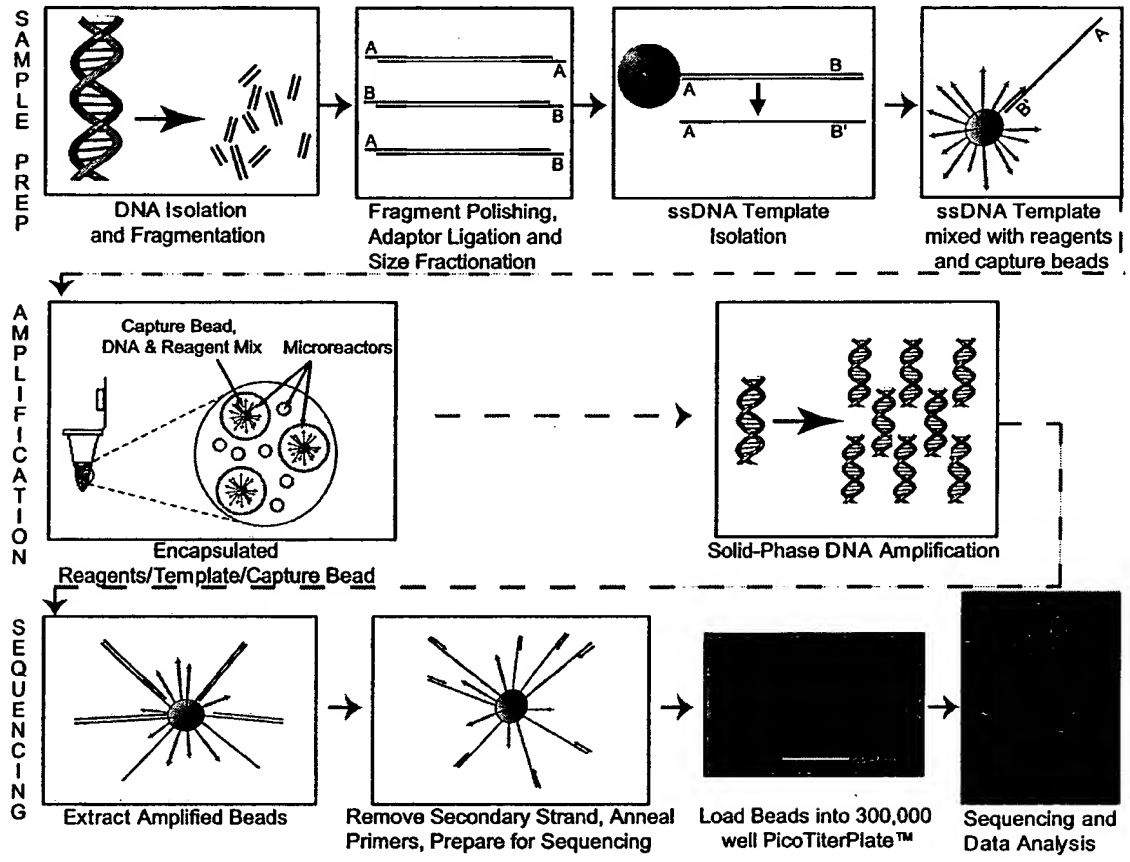

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[redacted]

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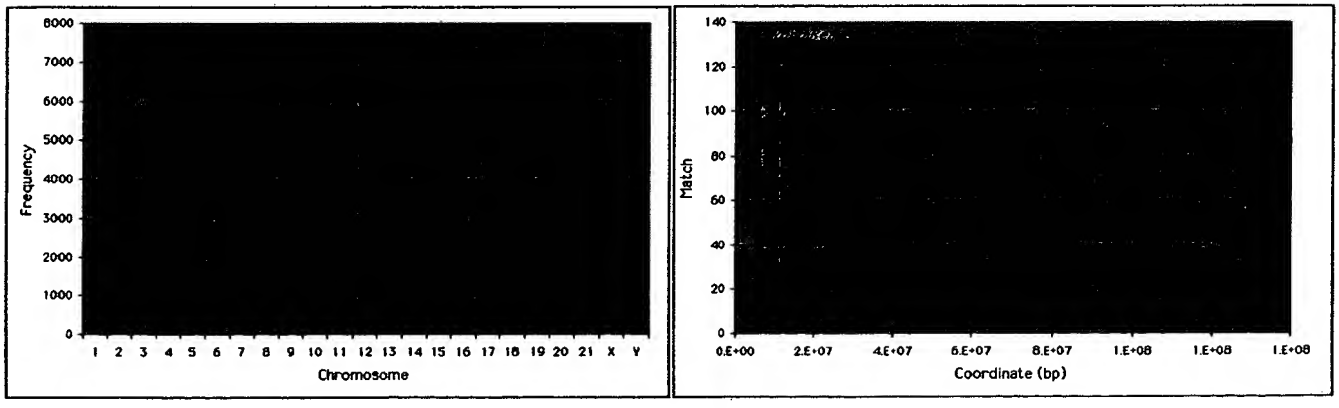
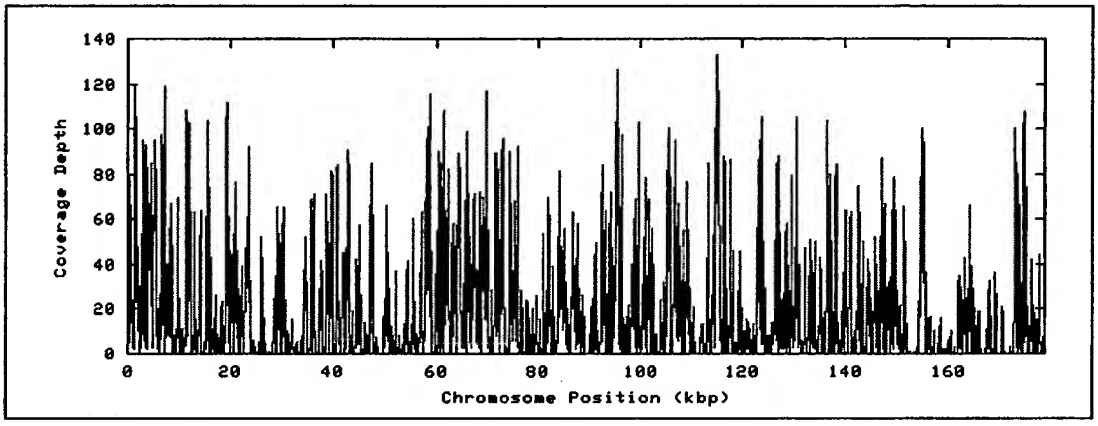


(a) Mapping against Human Genome (b) Mapping to Human Chr12

Figure 2. Sequence mapping against human genom and within chr mosome 12

Results and Discussion

BAC Assembly:
In a separate sequencing run, we generated 67193 raw reads from this BAC clone. After adaptor removal, repeat masking and quality trimming, 39900 reads were assembled against the reference sequence (Fig. 3). Genome coverage is 85% and consensus accuracy is 98%. Average read length is 84 bases.



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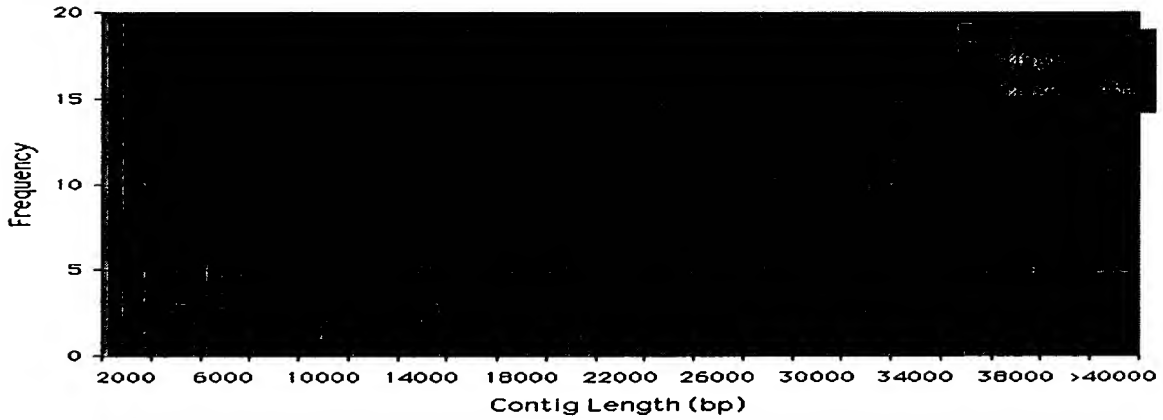

Figur 4. Distributi n f Phrap c ntigs fr m Sanger nly and Sanger+454 r ads

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